X099 Ligate & Electroporate 2-4Kb + >4Kb cDNA

Purpose: Make sure the larger sized fractions are okay & determine their sizes

Ligation

tube	vector	insert	H_2O	45°C 5'	1µl 10X lig buffer + 1µl T4 DNA Ligase
1	pcDNA1 NotI/BstXI gel purified X070 1µl = 24ng ✓	—	8µl ✓	✓	1µl
2	" ✓	cDNA 2-4Kb X095 1.5µl = 6ng ✓	6.5µl ✓	✓	1µl
3	" ✓	cDNA >4Kb X095 6µl = 6ng ✓	7µl ✓	✓	1µl ✓

15°C overnight

Attachment A

X099 cont'd

CREATION OF EXPRESSION LIBRARY POOLS

~~DAY 1~~:

Prepare bacteria

1) ~~Streak out MC1061/P3 onto LB/Kanamycin (15 µg/ml) plate~~

Prepare reagents and everything else

2) Make LB/Amp (15 µg/ml)/Tet (8 µg/ml) plates (15 cm). Make 1 liter total.
3) Sterilize:
   eppendorfs (1/electroporation)
   ddH20 (500 ml)
   10% glycerol (500 ml)
   pasteur pipets (long ones)
   1 M MgCl2
   1 M MgSO4
   1 M glucose
4) Put in cold room:
   all the above sterilized stuff except medium stuff
   electroporation cuvettes (0.2 cm gap)
   pipet tips (yellows/blues?)
   also sign up centrifuges

~~DAY 2~~:

Bacteria

8) Start overnight cultures of MC1061/P3 from A. Pearsons plate of MC1061/P3 from Lodish lab
   Pick ~~at least 2 colonies~~ into 3 mls each of LB + Kanamycin (15µg/ml)
   ~~Also streak each onto half a plate of LB/Amp/Tet (only undesirable revertents should grow)~~

~~DAY 3~~:

Get bacteria ready for electroporation

9) Put 3 ml of starter culture into 250 ml LB + Kanamycin (15µg/ml)
   Grow until 0.5 - 0.7 O.D.

time	O.D.600
7:25	-
10:00	0.320
10:20	0.497
10:35	0.600 ⇒ on ice

Clean up ligation

10) Add TE pH 8.0 to 50 µl
11) Add 50 µl Phenol/Chloroform/Isoamly alcohol
    Vortex, Spin and recover top aqueous layer
12) Add 50 µl TE pH 8.0 to organic layer to backextract
    Vortex, Spin and recover top aqueous layer and add to previous aq. layer (total = 100µl)
13) Add
    ~~10~~ 5 µl 1X LPA ✓
    10 µl 3M NaOAc ✓
    ~~250~~ 250 µl 100% ethanol
14) Put at -80°C 30 min 8:30
15) Spin down at 4°C, remove supe and ~~air dry~~ speed-vac (don't dry completely) — rinse w/ 70% EtOH — tube I dried completely
16) ~~Just before ready to use,~~ resuspend in 8 µl TE (sterile) keep on ice

Use 2µl/electroporation
Freeze rest in cDNA box -20°C.

Get bacteria ready for electroporation (everything on ice!)

17) Put culture into ice water to chill 15 min (swirl occasionally) 10:35 - 10:50
18) Spin down in 1 disposable conical tube, 4°C, 15 min, 4000 rpm (2600 xg), 10:55 - 11:10
19) Decant most but not all liquid (leave equal volume liquid as in pellet). Add 5 ml sterile water and resuspend gently with pipet.
20) Add 250 ml ice-cold ddH20 (sterile), spin 15 min, 4°C, 4000 rpm
21) Repeat steps 19-20 but spin 20 min..
22) Pour off as much supe as possible (you'll lose some bugs), add 10% glycerol to 12 mls, gently resuspend cells and spin 8,000 rpm 30 min 4°C in SS-34 (in Falcon 2059 tube)
23) Pour off supe getting rid of almost all liquid (you'll lose some cells). You want it thick. Resuspend in 100 µl 10% glycerol (you want it thick) - Used 200µl had 100µl left over Next time resuspend in 50µl? or don't add any liquid at all? yes should get only 3 electro/250ml

During spin periods, set up for electroporation

24) Make SOC from SOB
25) Put electroporator chamber on ice
26) Connect pulse controller to gene pulser (connect in front the red to red and black to black). The cuvette holder should then be connected to the pulse controller.
27) Set to:
    200 ohms
    25 µF
    2.5 kV
28) Get everything else ready (Falcon 2059 with 1 ml SOC each, pasteurs, tips, etc)

Electroporation

Always do controls: water only (neg. control) and uncut vector (positive control)

30) Swirl bacteria with sterile yellow tip. Pipet up 40 µl bacteria to tube #1 on ice. Pipet up and down avoiding generation of bubbles. Let sit 30 sec on ice.
31) With fresh tip take up bacteria and put into cuvette as close to bottom as possible without creating bubbles. Quickly shake hard down to bottom (v. important).
32) Take off cap, put in electroporator chamber, pulse
33) Quickly remove cuvette and add 1 ml SOC. Resuspend with pasteur pipet and transfer to 15 ml round bottom and incubate shaking at 37°C, 60 min. 1pm - 2pm
34) Repeat steps - for each electroporation.
35) Put LB/Amp/Tet plates into hood to dry.
36) Plate out 50 - 100 µl/plate to test for electroporation efficiency.
    Use 1:100 of positive control
    ~~1:5 of ligation mix~~
    undiluted neg. control
    Grow overnight 37°C. Store electroporated bacterial cultures at 4°C up to one week.
37) Count colonies.

electro #	DNA	tau	amt plated	colonies	effic. (col/ug)	For 5000 col per plate vol of undi
1	Lig #1 - 2µl of 8µl	4.5	10µl + 90µl LB	70	1.2×10^6	–
2	0 #2 "	4.5	↓	2072	3.5×10^7	24µl
3	" #3 "	4.5	↓	1300	2.2×10^7	38µl
4	- (2µl TE)	4.5	100µl	13	–	–
5	pcDNA1 insert (1µl = 0.5ng) 2µl	4.5	1µl + 99µl LB	555	5.6×10^8	–

electro

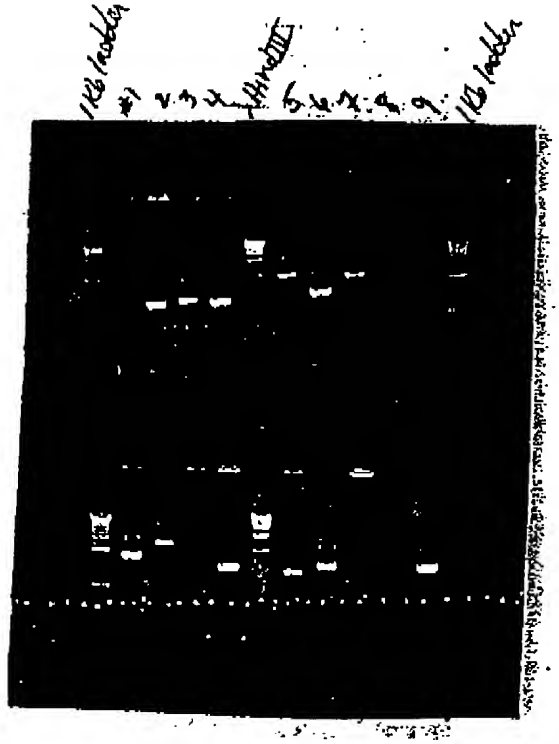
#2 pcDNA1 + 2.4Kb 24µl/plate ⇒ 42 plates = 210,000 clones assume 75% loss - 18ng/plate ~32 plates
#3 pcDNA1 + >4Kb 38µl/plate ⇒ 26 plates = 130,000 clones 18ng/plate ~20 plates

X099 cont'd

PCR of colonies to check cDNA sizes

19μl 10X PCR buffer
47.5μl T7 primer
47.5μl SP6 primer
0.95μl Taq polymerase
1.52 mix of dNTPs (each 25mM) → 3μl dATP, 3μl dCTP, 3μl dTTP, 3μl dGTP
73.5μl H_2O
190μl total
12μl dNTPs (freeze in PCR box -20°C)

Aliquot 15μl PCR oil/tube
Aliquot 10μl above stock soln/tube
Flame straight needle, poke colony, then into PCR tube
PCR 94°C 30 sec → 50°C 30 sec → 72°C 2min. x35 cycle
Add 1μl 10X blue juice
Load 6μl/lane onto 0.9% SeaKem GTG agarose minigel



colony	cDNA size	minigel 2.5
1	–	
2	0.7	
3	0.8	
4	0.7	
5	2.0	
6	1.0	
7	1.9	
8	–	2.1
9	–	2.8
10	1.2	
11	2.4	
12	–	0.8
13	0.9	
14	0.8	
15	1.0	
16	–	3.0
17	–	–
18	0.9	

avg size = 1.6
med size = 1.9

avg. size = 1.4
med size = 0.95

X099 cont'd

Mini-preps of clones that did not PCR

Method: Maniatis

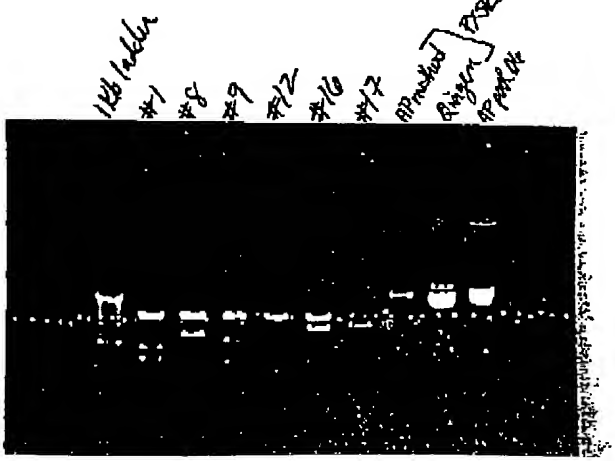
Changes: ① spun twice to get rid of white particulate matter after adding sol'n III
② Phenol/Sevag extracted
③ Resuspended in 25µl TE pH 8

original cDNA fraction	Colonies
2-4Kb	1, 8, 9
>4Kb	12, 16, 17

Digest plasmid ~~2µl~~ + NEB2 10x buffer 7µl + 10X AcBSA 7µl + ~~200 ng/µl~~ 10 mg/ml RNAse 0.1µl + H_2O ~~25~~ µl + No+I+HindIII 50:50 mix 7µl

2µl each + 8µl above rxn mix

37°C 2hrs (3:45-5:45)



colony	size
#1	1.1+1.4=2.5
8	2.1
9	1.0+1.8=2.8
12	0.8
16	3.0
17	–

PX5R3 (1µl each) - Not sure if state of DNA is very different or loading made a difference in the way it ran

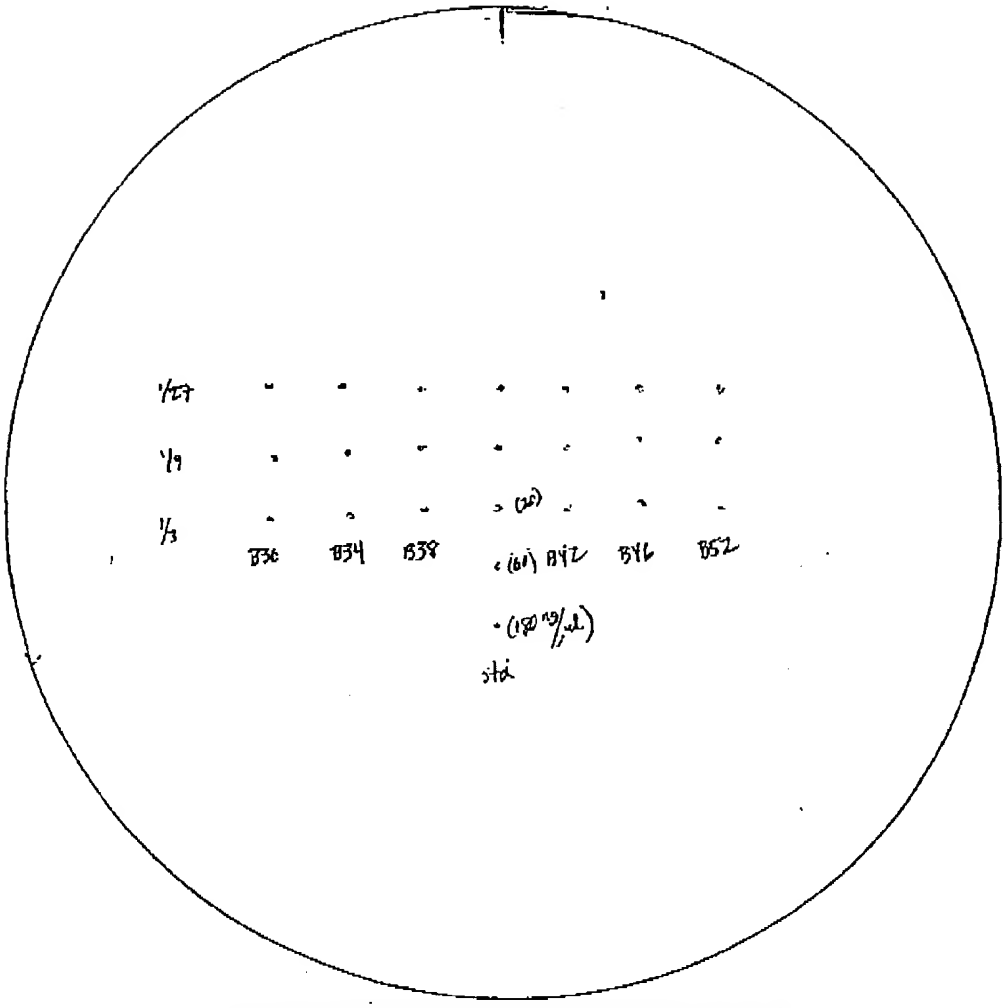
Plasmid midiprep for cDNA library

preps: B46 - B53

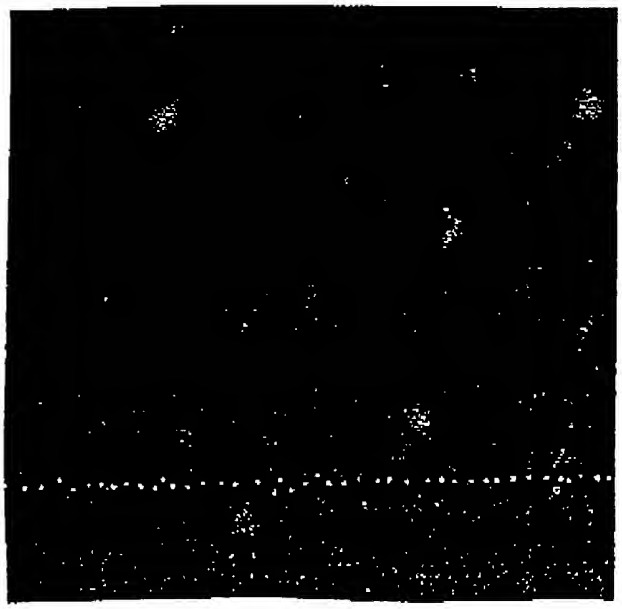
Day 1

1. Scrape 150 mm plate with 5 mls LB. Transfer to Falcon 2059 15 ml tube on ice.
2. Add another 3 mls LB to plate and scrape again.
3. Take 400 µl, put into freezer vial, add 100 µl glycerol and freeze at -150C.
4. Spin rest in SS-34, 9000 rpm, 2 min 4C.
5. Dry pellet as much as possible.
6. Resuspend pellet in 500 µl ice cold solution I by vigorous vortexing.
7. Add 1 ml fresh solution II (0.2 N NaOH, 1% SDS)
   for 100 ml: 1 ml 2 N NaOH
   0.5 ml 20% SDS
   8.5 mls ddH20
   Swirl gently until clear. Do not vortex. Leave on ice 10 min.
8. Add 750 µl solution III (ice-cold). Close tube and mix contents by shaking vigorously several times. Store on ice 5'. A flocculent white precipitate should form.
9. Centrifuge 15', 4°C, 9000 rpm.
10. Recover supe and add equal volume of phenol:chloroform. Mix by vortexing.
11. Spin 9000 rpm, 5'.
12. Add 2 volumes, ethanol r.t., vortex, let stand 5'.
    Spin in SS-34 rotor for 15' 9,000 rpm.
13. Carefully aspirate off all liquid (want it dry) and rinse once in cold 70% EtOH. Aspirate again getting any extraneous drops. Airdry 10' minutes.
14. Redissolve in 100 µl TE plus DNAse-free RNAse (20 µg/ml). Vortex briefly. ~~Incubate 37C, 30'.~~
    Transfer to sterile eppendorf.
15. Quantitate by dilutions onto EtBr plate.

Store at 4°C O.N.
Incubate 37°C 2½ hrs
Freeze



Note:
photograph is mirror image of schematic above



Slight variation in preps. I think 5 ul/transfection will be good for all.

X112 Screen cDNA pools B63-B76, redo B47

DEAE dextran transfections of COS M6 cells

materials:

1. 35 mm dishes.
2. DMEM with 10% FBS
3. Chloroquine (40 mM in CMF PBS, sterile filtered)
4. DNA
5. CMF PBS
6. DEAE-dextran (10 mg/ml in CMF PBS (autoclaved))
7. DMSO
8. cPBS
9. sterile tips

method:

day 0 (set up cells)

Set COS M6 cells in 35 mm dishes at 300,000 cells/dish in 2 ml DMEM with 10% FBS

day 1 (transfect)

1. In sterile eppendorfs prepare for each dish add (in order):
   a) DNA - 500 ng/dish
   b) add CMF PBS to 190 µl, vortex
   c) 10 µl of 10 mg/ml DEAE-dextran, vortex well (7 seconds)

tube #	plates	DNA			CMF PBS	10 mg/ml DEAE-dextran	positives	mayb
1		cDNA pool	B63	10µl	180µl	10µl	1	2
2			B64				4	7
3			B65				1	1
4			B66				1	0
5			B67				2	0
6			B68				0	1
7			B69				0	4
8			B70				2	3
9			B71				2	4
10			B72				0	1
11			B73				4	0
12			B74				12	4
13			B75				2	5
14			B76				2	2
15			B47 - redo from X111				9	4
16			B47 - redo from X111				12	2
17		pcDNA1		0.39			0	1
18		1:5000		9.2µl			396	8

2. Rinse cells with 2 mls CMF PBS (37C). Aspirate PBS.
3. Add transfection cocktail from step 1 and distribute evenly. Incubate 30 minutes, distributing liquid every 10 min. 9:50 - 10:20
4. Add 2 ml DMEM 10% FBS + 80 µM chloroquine and incubate 37C 2.5 hrs. 10:20 - 12:50
5. Aspirate off medium and replace with 1 ml 10% DMSO in DMEM 10% FBS for 2.5 min.
6. Aspirate off and wash once with 2 mls cPBS.
7. Refeed with 2 ml warm DMEM 10% FBS/dish. Incubate overnight.

Day 2

Refeed cells with 2 ml DMEM 10% FBS + 1 mM Nabutyrate pH 7.3.

Day 3

Refeed cells with 0.75 ml DMEM 10% FBS + 1 mM Nabutyrate pH 7.3 + 3 µg/ml DiI-AcLDL for 5 hrs.

14.25 ml DMEM 10% + 14.25µl 1M Nabut + 158µl DiI-AcLDL prep #48 (0.27 mg/ml)

plate	DNA	positives	mayhew
(1)	B47 pool	7 (10)	5 (6)
2	B48	3	4
3	B49	1	0
4	B50	2	6
5	B51	0	1
6	B52	0	0
7	B53	2	2
8	B54	2	5
9	B55	0	0
10	B56	2	4
11	B57	2	5
12	B58	3	8
13	B59	1	4
14	B60	3	2
(15)	B61	6 (4)	2 (3)
16	B62	1	2
17	pcDNA1	1	2
18	1:5000	430	–

Positive are scored if cells are punctate
parenthesis are recounts

X120 Create subpools of 15 colonies of B47.1.8

Purpose: Reduce pool size to approx 15 colonies to narrow the search for the MAC26-1 receptor.

Transform competent MC1061/P3 (Q.G. purple dot) usual procedure but didn't incubate on ice 30'; just heat-shocked 37° 5' right away. Still worked

Plated 5µl - count 930 (B47.1.8)
4 (no DNA)

Took 3.2µl transformed bugs + 1.9 mls LB
plated 50µl/plate

Counted plates

	colonies
B47.1.8.1 -	19
.2 -	15
.3 -	19
.4 -	23
.5 -	15
.6 -	13
.7 -	17
.8 -	21
.9 -	15
.10 -	33
.11 -	18
.12 -	17
.13 -	22
.14 -	26
.15 -	11
.16 -	16
.17 -	17
.18 -	15
.19 -	25
.20 -	14
.21 -	19
.22 -	16
.23 -	23
.24 -	23
.25 -	35
.26 -	16
.27 -	19
.28 -	23
.29 -	26
.30 -	22
.31 -	19
.32 -	18
.33 -	17
.34 -	20
35 -	19
36 -	31

1st 24 pools	all 36 pools
452	717
plate 18.8	19.9

IX120 cont'd

Plasmid midiprep for cDNA library

preps: B47.1.8.1 - B47.1.8.24

Day 1

1. Scrape 100 mm plate with 2 mls LB. Transfer to Falcon 2059 15 ml tube on ice.
2. Add another 2 mls LB to plate and scrape again.
3. Spin in SS-34, 9000 rpm, 2 min 4C.
5. Dry pellet as much as possible.
6. Resuspend pellet in 300 µl ice cold solution I by vigorous vortexing.
7. Add 0.6 ml fresh solution II (0.2 N NaOH, 1% SDS)
   for 100 ml: 1 ml 2 N NaOH
   0.5 ml 20% SDS
   8.5 mls ddH20
   Swirl gently until clear. Do not vortex. Leave on ice 10 min.
8. Add 450 µl solution III (ice-cold). Close tubeand mix contents by shaking vigorously several times. Store on ice 5'. A flocculent white precipitate should form.
9. Centrifuge 15', 4°C, 9000 rpm.
10. Recover supe and add equal volume (1.2 ml) of phenol:chloroform. Mix by vortexing.
11. Spin 9000 rpm, 5'.
12. Add 2 volumes (2.5) ethanol r.t., vortex, let stand 5'. overnight (1-16) or 6hrs (17-24)
    Spin in SS-34 rotor for 15' 9,000 rpm.
13. Carefully aspirate off all liquid (want it dry) and rinse once in cold 70% EtOH. Aspirate again getting any extraneous drops. Airdry 10' minutes.
14. Redissolve in 50 µl TE plus DNAse-free RNAse (20 µg/ml). Vortex briefly. Incubate 37C, 2 hr. Transfer to sterile eppendorf.
15. ~~Quantitate by dilutions onto EtBr plate.~~

X121 Screen subpools B47.1.8.1 - B47.1.8.24; compare CD36 with B47.1.8

DEAE dextran transfections of COS M6 cells

Method:

Day 0 (set up cells)

Set COS M6 cells in 35 mm dishes at 300,000 cells/dish in 2 ml DMEM with 10% FBS

Day 1 (transfect)

1. In sterile eppendorfs prepare for each dish add (in order):
   a) DNA - 500 ng/dish
   b) add CMF PBS to 190 µl, vortex
   c) 10 µl of 10 mg/ml DEAE-dextran, vortex well (7 seconds)

tube	# plates		CMF PBS	10 mg/ml DEAE-dextran	Results
1	~~B47.1.8.1~~	10µl	180µl	10µl	−
2	2				−
3	3				−
(4)	4				+ ?
5	5				−
6	6				−
7	7				−
8	8				−
9	9				−
10	10				−
11	11				−
12	12				−
13	13				−
14	14				−
15	15				−
16	16				−
17	17				−
18	18				−
(19)	19				+
20	20				−
21	21				−
22	22				−
23	23				−
(24)	24				−
25	bSR2 (pSR3)	-	1.5µl	570µl	30µl + v. bright
26	↓	poly I	X	X	X nothing
27	↓	m.BSA	X	X	X v. bright
28	CD36	-	15µl	525µl	30µl v. bright
29	↓	poly I	X	X	X v. bright
30	↓	m.BSA	X	X	X nothing ?
31	B47.1.8	-	30µl	540µl	30µl pos. cells
32	↓	poly I	~~30µl~~ X	X	X pos. cells
33	↓	m.BSA	X	X	X nothing ?
34	pcDNA1		0.3µl	190µl	10µl
~~35~~					

Pool #4 was brightest & w/more positive cells.

CD36 binds acetylated LDL & is not inhibited by poly I but is inhibited by small amounts of m-BSA.

∴ CD36 has same properties as MAC 26-1 receptor!

X121 cont'd

2. Rinse cells with 2 mls CMF PBS (37C). Aspirate PBS.
3. Add transfection cocktail from step 1 and distribute evenly. Incubate 30 minutes, distributing liquid every 10 min.
4. Add 2 ml DMEM 10% FBS + 80 µM chloroquine and incubate 37C 2.5 hrs. 11:50 - 1:20 pm
5. Aspirate off medium and replace with 1 ml 10% DMSO in DMEM 10% FBS for 2.5 min.
6. Aspirate off and wash once with 2 mls cPBS.
7. Refeed with 2 ml warm DMEM 10% FBS/dish. Incubate overnight.

Day 2

Refeed cells with 2 ml DMEM 10% FBS + 1 mM Nabutyrate pH 7.3.

Day 3

Refeed cells with 0.75 ml DMEM 10% FBS + 1 mM Nabutyrate pH 7.3 + 3 µg/ml DiI-AcLDL for 5 hrs.

27 mls med + 27µl 1M Nabut + 300µl DiIAcLDL (#48 0.27mg/ml)

2.25 mls + 225µl polyI (4mg/ml) = 400µg/ml

2.25 mls + ~~1~~ 1.35 m-BSA (3.34mg/ml) = 2µg/ml

9:20 - 2:20 pm

# pos cells	brightn
4 > 24 > 19	4 > 24 > 19

XIZZ Create subpools of 1 colony of B47.1.8 and Screen

Purpose:

Transform competent (CaCl2) MC1061/P3 (Q.One purple)

1. thaw aliquots (2) of bugs on ice
2. Add 2µl of DNA (or TE) to aliquot
   DNA = pool B47.1.8.4
   TE = neg. control
3. Carefully pipet up & down twice to mix
4. Heat shock 37°C 5 min.
5. Add 200µl LB medium Shake 1hr 37°C
6. Plate 5µl on 150mm LB Amp/1 et plate

Results: Transformation worked well. Circled 49 apparently single colonies to be picked. Picked each (done by me ~~Ana Maria Vrinceanu~~) into 3 mls LB A/T. Grew overnight.

Also had Ana Maria Vrinceanu repick off same plate & streak out to new plates. (4 indiv/150mm plate)

X122 cont'd

Plasmid miniprep for cDNA library

preps: Matrix. 7×7 rows A-F, columns 1-7

14 mini-preps

Day 1

1. Take 200 µl culture from each tube in a row or column of 7 and put into eppendorf. Store remainder at 4°C.
2. Spin at 12,000 x g for 30 sec in microfuge.
3. Remove medium by aspiration, leaving bacterial pellet as dry as possible.
4. Resuspend pellet in 100 µl ice-cold solution I by vigorous vortexing.
5. Add 200 µl fresh solution II (0.2 N NaOH, 1% SDS)
   for 2 ml: 0.2 ml 2 N NaOH
   0.1 ml 20% SDS
   1.7 mls ddH20
   Swirl gently until clear. Do not vortex. Leave on ice 10 min.
6. Add 150 µl solution III (ice-cold). Close tube and vortex gently inverted for 5 sec. Store on ice 5'. A flocculent white precipitate should form.
7. Centrifuge 5', 4°C, max speed in microfuge: 400µl
8. Recover supe and add equal volume of phenol:chloroform. Mix by vortexing.
9. Spin 2' in microfuge. 800µl
10. Add 2 volumes, ethanol r.t., vortex, let stand 2' at r.t.
    Spin 5', 4°C max speed in microfuge.
11. Carefully aspirate off all liquid (want it dry) and rinse once in cold 70% EtOH. Aspirate again getting any extraneous drops. Airdry 10' minutes.
12. Redissolve in 10 µl TE plus DNAse-free RNAse (20 µg/ml). Vortex briefly. Incubate 37C, 0.5 hr. ~~Transfer to sterile eppendorf.~~

X122 cont'd Screen matrix

DEAE dextran transfections of COS M6 cells

materials:

1. 35 mm dishes
2. DMEM with 10% FBS
3. Chloroquine (40 mM in CMF PBS, sterile filtered)
4. DNA
5. CMF PBS
6. DEAE-dextran (10 mg/ml in CMF PBS (autoclaved))
7. DMSO
8. cPBS
9. sterile tips

method:

day 0 (set up cells) 6-well
Set COS M6 cells in ~~35~~ mm dishes at 300,000 cells/dish well in 2 ml DMEM with 10% FBS

day 1 (transfect)

1. In sterile eppendorfs prepare for each dish add (~~in order~~):
   a) DNA - 500 ng/dish
   b) add CMF PBS to 190 µl, vortex
   c) 10 µl of 10 mg/ml DEAE-dextran, vortex well (7 seconds)

tube	# plates	DNA	CMF PBS	10 mg/ml DEAE-dextran	Results
1	row A	plasmid prep from matrix	5µl	185µl	10µl + 3rd brightest
2	B				−
3	C				−
4	D				+ v. faint flu.
5	E				−
6	F				+ 2nd brightest
(7)	(G)				+ brightest row
8	column (1)				+
9	2				−
10	3				+
11	4				+ weak
12	5				−
13	(6)				+ poor.
14	(7)				+ prob. brightest
15	pcDNA1		0.5	190µl	−
16	1:5000 (bsrGI)		9.3ml	180µl	
17					
18					

2. Rinse cells with 2 mls CMF PBS (37C). Aspirate PBS.
3. Add transfection cocktail from step 1 and distribute evenly. Incubate 30 minutes, distributing liquid every 10 min.
4. Add 2 ml DMEM 10% FBS + 80 µM chloroquine and incubate 37C 2.5 hrs.
5. Aspirate off medium and replace with 1 ml 10% DMSO in DMEM 10% FBS for 2.5 min.
6. Aspirate off and wash once with 2 mls cPBS.
7. Refeed with 2 ml warm DMEM 10% FBS/dish. Incubate overnight.

Day 2
Refeed cells with 2 ml DMEM 10% FBS + 1 mM Nabutyrate pH 7.3.

Day 3
Refeed cells with 0.75 ml DMEM 10% FBS + 1 mM Nabutyrate pH 7.3 + 3 µg/ml DiI-AcLDL for 5 hrs.

13.5 ml " + 13.5 µl 1M Nabut + 150 µl DiIAcLDL #48 (0.27 mg/ml)

9:20 - 2:20

Results: lots! of positives. Pool & expand brightest 50

X122 (cont'd) Matrix

	1	2	3	4	5	6	7
A	1	2	3	4	5	6	7
B	8	9	10	11	12	13	14
C	15	16	17	18	19	20	21
D	22	23	24	25	26	27	28
E	29	30	31	32	33	34	35
F	36	37	38	39	40	41	42
G	✓43	44	✓45 neg 7/10/03	46	47	✓48 neg 7/10/03	✓49

As stated before,
Plated all 49 out on LB Amp/Tet plates
(before results of this exp't were known)

Picked a single colony (hopefully) from the plates of #7, 28, 42, 43, 45, 46, 48, 49
#42 did not grow, but rest did
Decided to keep things simple so mini-preped only #43, 45, 48, 49 (see X124)

X124 cont'd

DEAE dextran transfections of COS M6 cells

materials:

1. 35 mm dishes
2. DMEM with 10% FBS
3. Chloroquine (40 mM in CMF PBS, sterile filtered)
4. DNA
5. CMF PBS
6. DEAE-dextran (10 mg/ml in CMF PBS (autoclaved))
7. DMSO
8. cPBS
9. sterile tips

method:

day 0 (set up cells) 6-well well

Set COS M6 cells in ~~35~~ mm dishes at 300,000 cells/dish in 2 ml DMEM with 10% FBS

day 1 (transfect) - Note: cells were way too heavy (used Coulter counter) Its better to use 1 confluent T75 for 18 wells than use the counter which isn't working.

1. In sterile eppendorfs prepare for each dish add (in order):
   a) DNA - 500 ng/dish
   b) add CMF PBS to 190 µl, vortex
   c) 10 µl of 10 mg/ml DEAE-dextran, vortex well (7 seconds)

tube	# plates		CMF PBS	10 mg/ml DEAE-dextran	results
1	847.1.8.4.43	- 5µl	185µl	10µl	+
2	.45				-
3	.48				-
4	.49				+ brighter
5	pcDNA1	- 0.5µl	190		-
6	PX3R3	- 0.5µl			+
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					

2. Rinse cells with 2 mls CMF PBS (37C). Aspirate PBS.
3. Add transfection cocktail from step 1 and distribute evenly. Incubate 30 minutes, distributing liquid every 10 min.
4. Add 2 ml DMEM 10% FBS + 80 µM chloroquine and incubate 37C 2.5 hrs.
5. Aspirate off medium and replace with 1 ml 10% DMSO in DMEM 10% FBS for 2.5 min.
6. Aspirate off and wash once with 2 mls cPBS.
7. Refeed with 2 ml warm DMEM 10% FBS/dish. Incubate overnight.

Day 2

Refeed cells with 2 ml DMEM 10% FBS + 1 mM Nabutyrate pH 7.3.

Day 3

Refeed cells with 0.75 ml DMEM 10% FBS + 1 mM Nabutyrate pH 7.3 + 3 µg/ml DiI-AcLDL for 5 hrs.

6 wells
6.5 mls

1M 0.27 mg/ml

4.85 ml MEM ... 1%

B47.1.8.4.49 was chosen as cloned MAC2L-1 receptor & was renamed phaSR III (Note: Ana Maria Vincenan did several exp'ts to show that the plasmid was a single one & repeatedly gave the expected activity)

X133 Sequencing of 5' end of pha8RIII w/ Sequenase kit

Purpose: Determine if pha8RIII encodes a known protein (e.g. CD36 or Limp II)

Followed kit instructions. Used primers:
T7 - on plasmid pcDNA1
05A1.3 - in cDNA sequence
5' CTG TCG CTG TCC CCC TTC AG 3'

T7

short read
GGTACCGAGCTCGATCCACTAGTAACGGCCGCCAGTGTGCTCTAAAGGCCACCTGCAGGGCTACTG
CTGCTCCGGCCACTGCCTGAGACTCACCTTGCTGGAACGTGAGCCTCGGCTTCTGTCATCTCTG

long read
ACTCACCTTGCTGGAACGTGAGCCTCGGCTTCTGTCATCTCTGTGGCCTCTGTCGCTTCTGTCGCT
GTCCCCCTTNAGTCCCTGAGCCCCGCGAGCCCGGGCCGCACACGCGACATGGGCGGCANNCCAGGG

mix
GGTACCGAGCTCGATCCACTAGTAACGGCCGCCAGTGTGCTCTAAAGGCCACCTGCAGGGCTACTG
CTGCTCCGGCCACTGCCTGAGACTCACCTTGCTGGAACGTGAGCCTCGGCTTCTGTCATCTCTGTG
GCCTCTGTCGCTTCTGTCGCTGTCCCCCTTNAGTCCCTGAGCCCCGCGAGCCCGGGCCGCACACGC
GCACATGGGCGGCANNCCAGGG

OSA3.1

short read
CCGCACACGCGACATGGGCGGCACGGCCAGGGCGCTGGGTGGCGGTGGGGCTGGGCGTCGTGGGGC
TGCTGTGCGCTGTGCTCGGTGTGGTTATGATCCTCGTGATGCCCTCGCTCATCAAACAGCAGGTAC
TGAAGAATGTC(C?)GCATAGACCCAGCAGCCTGTCTTTGCAATGTGGAAGGAGA

long read
TGTGCTCGGTGTGGTTATGATCCTCGTGATGCCCTCGCTCATCAAACAGCAGGTACTGAAGAATGT
CCGCATAGACCCCAGCAGCCTGTCCTTTGCAATGTGGAAGGAGATCCCTGTACCCTTCTACTTGTC
CGTCTACTTCTTCGAGGTGGTCAATCCCAGCGAGATCCTAAAGGGTGAGAA

mix T7 and OSA3.1
GGTACCGAGCTCGATCCACTAGTAACGGCCGCCAGTGTGCTCTAAAGGCCACC
TGCAGGGCTACTGCTGCTCCGGCCACTGCCTGAGACTCACCTTGCTGGAACGTG
AGCCTCGGCTTCTGTCATCTCTGTGGCCTCTGTCGCTTCTGTCGCTGTCCCCCTTN
AGTCCCTGAGCCCCGCGAGCCCGGGCCGCACACGCGACATGGGCGGCACGGCC
AGGGCGCTGGGTGGCGGTGGGGCTGGGCGTCGTGGGGCTGCTGTGCGCTGTGCT
CGGTGTGGTTATGATCCTCGTGATGCCCTCGCTCATCAAACAGCAGGTACTGA
AGAATGTCCGCATAGACCCCAGCAGCCTGTCCTTTGCAATGTGGAAGGAGATC
CCTGTACCCTTCTACTTGTCCGTCTACTTCTTCGAGGTGGTCAATCCCAGCGAG
ATCCTAAAGGGTGAGAA

BLASTX 1.3.9MP [Build

Reference: Gish, Warren and David J. States (1993). Identification of protein coding regions by database similarity search. Nature Genetics 3:266-72. Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers, and David J. Lipman (1990). Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Notice: statistical significance is estimated under the assumption that the equivalent of one reading frame in the query sequence codes for protein and that significant alignments will involve only coding reading frames.

Query= TITLE phasr3.seq
(447 letters)

Translating both strands of query sequence in all 6 reading frames

Database: Non-redundant PDP+SwissProt+PIR+SPupdate+GenPept+GPupdate, EDT
96,634 sequences; 27,090,059 total letters.
Searching.....done

Sequences producing High-scoring Segment Pairs:		Reading Frame	High Score	Smallest Poisson Probability P(N)	N
sp|P27615|LIM2_RAT	LYSOSOME MEMBRANE PROTEIN II (L...	+2	114	1.1e-08	1
pir|JQ1523|JQ1523	lysosomal membrane 85K sialogly...	+2	109	6.3e-08	1
sp|P10284|HM26_MOUSE	HOMEOBOX PROTEIN HOX-2.6. >pir|...	-2	61	2.4e-06	2
sp|P16671|CD36_HUMAN	PLATELET GLYCOPROTEIN IV (GPIV)...	+2	94	1.1e-05	1
gp|L06850|HUMCD36B_1	antigen CD36 [Homo sapiens]	+2	94	1.1e-05	1
gp|L19658|RATFAT_1	FAT gene product [Rattus norveg...	+2	92	2.3e-05	1
pir|A43932|A43932	mucin - human (fragment) | 0.0 ...	-1	60	3.8e-05	2
pir|B60492|B60492	homeotic protein Hox B4 - human...	-2	57	4.0e-05	2
sp|Q01200|PRIA_LENED	PRIA PROTEIN. >pir|S23106|S2310...	-1	62	5.6e-05	2
pir|S12968|S12968	Acrosin, sperm - Pig #EC-number...	-2	59	6.7e-05	2
gp|L23108|MUSCDANTI_1	CD36 antigen [Mus musculus]	+2	88	9.0e-05	1
pir|A45106|A45106	mucin - human (fragment) | 0.0 ...	-1	60	9.2e-05	2
pir|S31976|S31976	Cvx peptide - Rat | 0.0 0.0 0.0...	-3	57	0.00012	2
gp|Z16406|MOX2A_1	Mox-2 [Mus musculus]	-3	57	0.00012	2
gp|Z17223|RNGAXMR_1	Gax peptide [Rattus norvegicus]	-3	57	0.00012	2
sp|P13983|EXTN_TOBAC	EXTENSIN PRECURSOR (CELL WALL H...	-2	56	0.00024	2
pir|G60110|G60110	repetitive protein antigen 69/7...	-2	81	0.00035	1
gp|M14721|MUSFGNAA_1	Mouse epidermal profilaggrin mR...	-3	71	0.0044	1
pir|B36664|B36664	S59/4 homeotic protein - fruit ...	-3	76	0.0080	1

>sp|P27615|LIM2_RAT LYSOSOME MEMBRANE PROTEIN II (LIMP II) (85 KD LYSOSOMAL MEMBRANE SIALOGLYCOPROTEIN) (LGP85). >pir|A41180|A41180 74k lysosomal membrane protein LIMP - rat \| 0.0 0.0 0.0 0.0 0.0 >pir|JH0241|JH0241 85K lysosomal membrane sialoglycoprotein - rat \| 0.0 0.0 0.0 0.0 0.0 >gp|D10587|RATLGP85_1 LGP85 [Rattus sp.] >gp|M68965|RATLIMPII_1 lysosomal membrane protein [Rattus norvegicus]
Length = 478

```
 Plus Strand HSPs:

 Score = 114 (55.2 bits), Expect = 1.1e-08, P = 1.1e-08
 Identities = 22/64 (34%), Positives = 36/64 (56%), Frame = +2

Query:   254 LLCAVLGVVMILVMPSLIKQQVLKNVRIDPSSLSFAMWKEIPVPFYLSVYFFEVVNPSEI 433
             LL   + +++  V    + Q + KN+ +   + F  W++ P+P Y+  YFF V NP EI
Sbjct:    16 LLVTSVTLLVARVFQKAVDQTIEKNMVLQNGTKVFDSWEKPPLPVYIQFYFFNVTNPEEI 75

Query:   434 LKGE 445
             L+GE
Sbjct:    76 LQGE 79


>pir|JQ1523|JQ1523 lysosomal membrane 85K sialoglycoprotein precursor - human |
            0.0 0.0 0.0 0.0 0.0 >gp|D12676|HUMHLGP85_1 85kDa human lysosomal
            sialoglycoprotein [Homo sapiens]
            Length = 478

 Plus Strand HSPs:

 Score = 109 (52.8 bits), Expect = 6.3e-08, P = 6.3e-08
 Identities = 21/64 (32%), Positives = 35/64 (54%), Frame = +2

Query:   254 LLCAVLGVVMILVMPSLIKQQVLKNVRIDPSSLSFAMWKEIPVPFYLSVYFFEVVNPSEI 433
             LL   + +++  V    + Q + K + +   + +F  W++ P+P Y   YFF V NP EI
Sbjct:    16 LLVTSVTLLVARVFQKAVDQSIEKKIVLRNGTEAFDSWEKPPLPVYTQFYFFNVTNPEEI 75

Query:   434 LKGE 445
             L+GE
Sbjct:    76 LRGE 79


>sp|P10284|HM26_MOUSE HOMEOBOX PROTEIN HOX-2.6. >pir|A31757|A31757 homeotic
            protein Hox 2.6 - mouse | 0.0 0.0 0.0 0.0 0.0 >gp|M36654|MUSHOX26_1
            Mouse homeo box 2.6 (Hox-2.6) mRNA, complete cds. [Mus musculus]
            Length = 250

 Minus Strand HSPs:

 Score = 61 (29.7 bits), Expect = 0.72, P = 0.52
 Identities = 13/41 (31%), Positives = 19/41 (46%), Frame = -2

Query:   251 PRRPAPPPPSALAVPPMSRVRPGLAGLRDSRGTATEATEAT 129
             P  P PPPP    + P + V+P    L    G  +EA  ++
Sbjct:    75 PPPPPPPPPPPPGLSPRAPVQPTAGALLPEPGQRSEAVSSS 115

 Score = 60 (29.3 bits), Expect = 2.4e-06, Poisson P(2) = 2.4e-06
 Identities = 13/25 (52%), Positives = 13/25 (52%), Frame = -2

Query:   278 PHRAQRTAAPRRPAPPPPSALAVPP 204
             P   QR AA R P PPPP     PP
Sbjct:    59 PCTVQRYAACRDPGPPPPPPPPPPP 83


>sp|P16671|CD36_HUMAN PLATELET GLYCOPROTEIN IV (GPIV) (GPIIIB) (CD36 ANTIGEN).
           >pir|A30989|A30989 CD36 protein - human | 0.0 0.0 0.0 0.0 0.0
           >gp|M24795|HUMANTCD36_1 Human CD36 antigen mRNA, complete cds.
```

```
[Homo sapiens] >gp|M98398|HUMCD3613_1 antigen CD36 [Homo sapiens]
>gp|M98399|HUMCD3621_1 antigen CD36 [Homo sapiens]
Length = 472

 Plus Strand HSPs:

 Score = 94 (45.5 bits), Expect = 1.1e-05, P = 1.1e-05
 Identities = 18/64 (28%), Positives = 36/64 (56%), Frame = +2

Query:   245 VVGLLCAVLGVVMILVMPSLIKQQVLKNVRIDPSSLSFAMWKEIPVPFYLSVYFFEVVNP 424
            V+G + AV G +++ V   LI++ + K V ++  +++F  W +     Y   + F+V NP
Sbjct:    14 VIGAVLAVFGGILMPVGDLLIQKTIKKQVVLEEGTIAFKNWVKTGTEVYRQFWIFDVQNP 73

Query:   425 SEIL 436
              E++
Sbjct:    74 QEVM 77


>gp|L06850|HUMCD36B_1 antigen CD36 [Homo sapiens]
           Length = 472

 Plus Strand HSPs:

 Score = 94 (45.5 bits), Expect = 1.1e-05, P = 1.1e-05
 Identities = 18/64 (28%), Positives = 36/64 (56%), Frame = +2

Query:   245 VVGLLCAVLGVVMILVMPSLIKQQVLKNVRIDPSSLSFAMWKEIPVPFYLSVYFFEVVNP 424
            V+G + AV G +++ V   LI++ + K V ++  +++F  W +     Y   + F+V NP
Sbjct:    14 VIGAVLAVFGGILMPVGDLLIQKTIKKQVVLEEGTIAFKNWVKTGTEVYRQFWIFDVQNP 73

Query:   425 SEIL 436
              E++
Sbjct:    74 QEVM 77


>gp|L19658|RATFAT_1 FAT gene product [Rattus norvegicus]
           Length = 472

 Plus Strand HSPs:

 Score = 92 (44.5 bits), Expect = 2.3e-05, P = 2.3e-05
 Identities = 18/65 (27%), Positives = 36/65 (55%), Frame = +2

Query:   245 VVGLLCAVLGVVMILVMPSLIKQQVLKNVRIDPSSLSFAMWKEIPVPFYLSVYFFEVVNP 424
            V+G + AV G +++ V   LI++ + + V ++  +++F  W +     Y   + F+V NP
Sbjct:    14 VIGAVLAVFGGILMPVGDLLIEKTIKREVVLEEGTIAFKNWVKTGTTVYRQFWVFDVQNP 73

Query:   425 SEILK 439
              E+ K
Sbjct:    74 EEVAK 78


>pir|A43932|A43932 mucin - human (fragment) | 0.0 0.0 0.0 0.0 0.0
           >gp|M74027|HUMMUC2A_1 mucin [Homo sapiens]
           Length = 573

 Minus Strand HSPs:
```

```
 Score = 60 (29.1 bits), Expect = 1.4, P = 0.74
 Identities = 12/21 (57%), Positives = 14/21 (66%), Frame = -1

Query:   279 TTPSTAHSSPTTPSPTATQRP 217
             TTPS   ++ TTPSPT T  P
Sbjct:   377 TTPSPPPTTMTTPSPTTTPSP 397

 Score = 58 (28.1 bits), Expect = 3.8e-05, Poisson P(2) = 3.8e-05
 Identities = 12/20 (60%), Positives = 14/20 (70%), Frame = -1

Query:   285 IITTPSTAHSSPTTPSPTAT 226
             I TTPS   ++ TTPSPT T
Sbjct:   343 ITTTPSPPTTTMTTPSPTTT 362


>pir|B60492|B60492 homeotic protein Hox B4 - human | 0.0 0.0 0.0 0.0 0.0
           Length = 251

  Minus Strand HSPs:

 Score = 57 (27.8 bits), Expect = 2.9, P = 0.95
 Identities = 12/21 (57%), Positives = 12/21 (57%), Frame = -2

Query:   266 QRTAAPRRPAPPPPSALAVPP 204
             QR AA R P PPPP     PP
Sbjct:    63 QRYAACRDPGPPPPPPPPPPP 83

 Score = 56 (27.3 bits), Expect = 4.0e-05, Poisson P(2) = 4.0e-05
 Identities = 11/20 (55%), Positives = 12/20 (60%), Frame = -2

Query:   254 APRRPAPPPPSALAVPPMSR 195
             +PR PAPPP  AL   P  R
Sbjct:    90 SPRAPAPPPAGALLPEPGQR 109


>sp|Q01200|PRIA_LENED PRIA PROTEIN. >pir|S23106|S23106 priA protein - Shiitake
           mushroom | 0.0 0.0 0.0 0.0 0.0 >gp|X60956|LEPRIA_1 priA gene
           product [Lentinus edodes]
           Length = 258

  Minus Strand HSPs:

 Score = 62 (30.0 bits), Expect = 0.61, P = 0.46
 Identities = 13/31 (41%), Positives = 18/31 (58%), Frame = -1

Query:   318 TCCLMSEGITRIITTPSTAHSSPTTPSPTAT 226
             TCCL     +    TPS+AH + T  SP++T
Sbjct:    90 TCCLPKWPTSTPTPTPSSAHHTSTHTSPSST 120

 Score = 56 (27.1 bits), Expect = 5.6e-05, Poisson P(2) = 5.6e-05
 Identities = 13/33 (39%), Positives = 16/33 (48%), Frame = -1

Query:   276 TPSTAHSSPTTPSPTATQRPGRAAHVACAARAR 178
             TPS+     +TP P+AT   G   H   A  AR
Sbjct:   143 TPSSPSKPSSTPKPSATPNKGNGHHYKRAHVAR 175


>pir|S12968|S12968 Acrosin, sperm - Pig #EC-number 3.4.21.10 | 0.0 0.0 0.0 0.0
```

```
                0.0
                Length = 374

     Minus Strand HSPs:

   Score = 59 (28.8 bits), Expect = 1.6, P = 0.79
   Identities = 14/48 (29%), Positives = 24/48 (50%), Frame = -2

Query:    251 PRRPAPPPPSALAVPPMSRVRPGLAGLRDSRGTATEATEATEMTEAEA 108
              P++ +  PP AL+   + ++   L G   S G +   TE T++ E  A
Sbjct:    326 PQQVSAKPPQALSFAKLQQLIEALKGTAFSSGRSYYETETTDLQELPA 373

   Score = 56 (27.3 bits), Expect = 6.7e-05, Poisson P(2) = 6.7e-05
   Identities = 11/21 (52%), Positives = 12/21 (57%), Frame = -2

Query:    266 QRTAAPRRPAPPPPSALAVPP 204
              Q  + PR PAPPPP     PP
Sbjct:    294 QPGSRPRPPAPPPPPPPPPPP 314


>gp|L23108|MUSCDANTI_1 CD36 antigen [Mus musculus]
           Length = 473

   Plus Strand HSPs:

   Score = 88 (42.6 bits), Expect = 9.0e-05, P = 9.0e-05
   Identities = 17/65 (26%), Positives = 35/65 (53%), Frame = +2

Query:    245 VVGLLCAVLGVVMILVMPSLIKQQVLKNVRIDPSSLSFAMWKEIPVPFYLSVYFFEVVNP 424
              V+G + AV G +++ V   LI++ + + V ++  + +F  W +     Y   + F+V NP
Sbjct:     15 VIGAVLAVFGGILMPVGDMLIEKTIKREVVLEEGTTAFKNWVKTGTTVYRQFWIFDVQNP 74

Query:    425 SEILK 439
              ++ K
Sbjct:     75 DDVAK 79
```